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ISOLATION, CLONING AND EXPRESSION OF THE GENES FOR MICROBIAL POLYURETHANE DEGRADATION.

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Office of Naval Research
800 North Quincy Street
Arlington, Virginia 22217-5000

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Authors--Fourth Report
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Project Scientist
Captain Steve Snyder

Abstract

Two fungi species, HAFB-2F-Br and HAFB-2F-Wh, have been isolated on polyurethane paint selective medium from Hill AFB paint waste samples. The mix removes polyurethane paint from the surface of painted plastic balls in less than 68 hours. Separately, HAFB-2F-Br strips the paint in 16 to 18 hours. Cloning has therefore begun with HAFB-2F-Br. Proteins have been extracted. The DNA vector and fungal host have been received. The pSV50 has been isolated and digested with *Bgl II* to remove the *cos* site and to reduce the number of *Bgl II* sites to one. The extracted linear plasmid DNA was ligated to itself to form a circular plasmid. Competent cells were prepared and the modified pSV50 was transformed into the competent cells. Several procedures have been used to extract the genomic DNA: the SDS approach was successful. Work is proceeding on further purification of the HAFB-2F-Br genomic library and plasmid isolation.

Identifiers/Open-Ended Terms
DNA/DNA libraries/16 S RNA sequence/
polyurethane paint/biodegradation/enzyme

Availability
Defense Technical Information Center
Bldg. 5, Cameron Station
Alexandria, Virginia 22314

Security Class
Unclassified
Unlimited

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Technical Research Associates would like to thank Captain Steve Snyder, Office of Naval Research, Arlington, Virginia, Project Scientist, for his support of the work. The time and effort he has put into the project is greatly appreciated.

FORWARD

This 6.1 Final Report covers work performed on Contract NOOO14-90-C-0182, entitled "Isolation, Cloning and Expression of the Genes for Microbial Polyurethane Degradation", technically from August 15, 1990 through May 31, 1991. This program was sponsored by the Office of Naval Research, 800 North Quincy Street, BCT #1, Arlington, Virginia 22217-5000. The Project Scientist was Captain Steve Snyder.

Mrs. Gail Bowers-Irons was both the Project Manager and Principal Investigator. Mr. Robert Pryor, Ms. Usha Charyulu, Ms. Sandra Haddad and Dr. Ramesh Prakash, all U.S. citizens, were responsible for the 6.1 project's experimentation.

SUMMARY

6.1/6.2 Technical Objectives:

- Purify the DNA from the TRA polyurethane paint degrading organism by density gradient centrifugation.
- Construct chromosomal DNA libraries via partial restriction endonuclease digestion and Cosmid cloning.
- Clone the plasmid DNA and perform restriction map analysis.
- Isolate the 16 S rRNA genes from the chromosomal DNA libraries.
- Initialize characterization and identification of the polyurethane paint digesting culture.

6.1/6.2 Work Statement:

The work in this project will be divided into five tasks. Task I will purify the DNAs by ethidium bromide (EtBr)-cesium chloride (CsCl) density gradient centrifugation. Task II will construct chromosomal DNA libraries. Task III will clone the plasmid DNA fragments. If time permits, Task IV work will isolate the 16 S rRNA genes from the chromosomal DNA libraries. Task IV will be run concurrently with Tasks I-III. The polyurethane paint-digesting culture will be initially characterized and identified.

6.1/6.2 Approach:

This project will focus on the isolation of DNA from TRA's polyurethane paint degrading culture. The work will search for and isolate established plasmids to determine if the plasmids are involved in the degradation process. DNA libraries will be created. Time permitting, the 16 S rRNA gene will be obtained using the DNA library gene(s). Concurrently, initial experiments will characterize the organisms. This approach provides background for future experiments, i.e., if the degradation of the paint is mediated by isolatable enzymes, the tryptic peptides could be sequenced; oligonucleotides created and DNA libraries screened for the responsible gene(s).

INTRODUCTION

Under new EPA standards, more efficient, less costly and less hazardous paint stripping techniques are needed. Technical Research Associates, Inc. (TRA) in an Air Force SBIR Phase I, successfully stripped and degraded polyurethane and epoxy paints with mixed microbial cultures. Deliberate, controlled and safe biodegradation of paint is possible.

This technology is both militarily and industrially important. Combat vehicles and logistics equipment, automobiles, commercial airplanes, ships, buildings--all materials which are painted--require constant repair and maintenance. There is also a need for hazardous waste or pollution control in Air Force facilities and EPA Superfund Site areas. Both enzymatic and microbial stripping systems could be self-contained and completely recycled. Commercial enzyme systems could be developed which would enable field level touch-ups, efficient corrosion control or complete degradation. The degradation would be less costly, toxic, and time consuming than current methods.

This project focuses on the isolation of DNA from TRA's polyurethane paint degrading microorganisms. Two fungi species, HAFB-2F-Br and HAFB-2F-Wh, have been isolated on polyurethane paint selective medium from Hill AFB paint waste samples. The mix removes polyurethane paint from the surface of painted plastic balls in less than 68 hours. Separately, HAFB-2F-Br strips the paint in 16 to 18 hours. Cloning has therefore begun with HAFB-2F-Br. Proteins have been extracted. The DNA vector and fungal host have been received. The pSV50 has been isolated and digested with *Bgl II* to remove the *cos* site and to reduce the number of *Bgl II* sites to one. The extracted linear plasmid DNA was ligated to itself to form a circular plasmid. Competent cells were prepared and the modified pSV50 was transformed into the competent cells. Several procedures have been used to extract the genomic DNA: the SDS approach was successful. Work is proceeding on further purification of the HAFB-2F-Br genomic library and plasmid isolation.

PROCEDURES

MATERIALS

Chemical	Manufacturer	Cat #	Lot#
Acetic Acid, Glacial	Fisher Scientific	A38s	882475
Agar	Difco	0138-01-4	773112
Agarose	FMC	50101	829790
Agarose	Fisher Scientific	BP-164	884370
Agarose - GTG	U.S. Biochemical	32827	70664
Ammonium Acetate	Fisher Scientific	A637	883008
Ammonium Nitrate	Fisher Scientific	A676	733055
Ammonium Sulfate	Fisher Scientific	A701	901675
Ampicillin	Sigma	A2804	110H-6730
Beef Extract	Sigma	B4888	68F-0252
Biotin	Fisher Scientific	BP232	865253
Boric Acid	Fisher Scientific	A74	872575
Bromophenol Blue	Aldrich	11,439-1	06026JW
Buffer Sol'n pH 10	Fisher Scientific	SB115	900236-24
Buffer Sol'n pH 4	Fisher Scientific	SB101	903467-24
Buffer Sol'n pH 7	Fisher Scientific	SB107	904254-24
Calcium Carbonate	Aldrich	25650-1	HX01920DX
Calcium Chloride	Baker	1-1332	116005476
Chloroform	Fisher Scientific	BP1145	911183
$CoSO4 \cdot 7H2O$	Aldrich	23038-3	EX02621TW
m-Cresol	Sigma	C5015	78F3478
$CuSO4 \cdot 5H2O$	Baker	1-1843	120000227
Dextrose	Fisher Scientific	D16	897594
DNA Dipstick™	Invitrogen	K5632-01	110307
EDTA Disodium Dihydrate	U.S. Biochemical	15699	67854
EDTA Disodium Salt	Mallinckrodt	4931	KXGD
Ethidium Bromide	Sigma	E7637	60H3402
Ethyl Alcohol, Absolute 200 proof	Quantum Chemical		
$FeSO4 \cdot 7H2O$	Fisher Scientific	I146	882789
Ficoll (MW 400,000)	Sigma	F-2637	99F-6750
GelGro®	ICN Biochemicals	150180	26398
ß-Glucuronidase/Arylsulfatase	Boehringer Mannheim	127698	1224182182
Glycerol	Fisher Scientific	BP229	881437
Hydrochloric Acid	Fisher Scientific	A144	903107
Impranil® (Polyurethane)	Mobay	D762	06Y08937
Iso-Amyl Alcohol	Fisher Scientific	BP1150	9111110
Isopropyl Alcohol	Fisher Scientific	A516	854124
Lambda DNA - Hind III	N.E BioLabs	301-2S	84
Lambda DNA - Hind III	Promega	G171A	60802
Lambda DNA - Hind III	Boehringer Mannheim	236250	1241172158
n-Lauroyl Sarcosine	Sigma	L5125	39F-0712
Lauryl Sulfate (SDS)	Sigma	L4390	70H-089415
Ligase, T4 DNA	Boehringer Mannheim	481220	1210822553
Lysing Enzymes (Novozyme234)	Sigma	L2265	60H0798

Lysozyme	Sigma	L6876	89F8275
$MgCl_2 \cdot 6H_2O$	Fisher Scientific	M-33	860876
MgO	Aldrich	24338-8	AX09908TW
$MgSO_4 \cdot 7H_2O$	Mallinckrodt	6066	KAL
$MnSO_4 \cdot H_2O$	Aldrich	22128-7	DX00619DV
NovoZym™ 234	Novo BioLabs	7367147	PPM3276
Penicillin G, Potassium	Gibco	8601840MJ	85K7401
Peptone	Difco	0118-01-8	787895
Phenol	U.S. Biochemical	20078	71113
Phosphatase, Alkaline (CIAP)	Boehringer Mannheim	13023	1230792230
Potassium Acetate	Fisher Scientific	BP364	884389
Potassium Phosphate (monobasic)	Mallinckrodt	7100	BKM
Proteinase K	Sigma	P4914	40H-0211
Restriction Enzymes:			
Bam HI	U.S. Biochemical	70520	72320
Bgl II	Boehringer Mannheim	348767	1217522070
Eco RI	U.S. Biochemical	70555	72321
Hind III	U.S. Biochemical	70570	72319
Sau 3A	Boehringer Mannheim	709743	1244452057
Ribonuclease A	Sigma	R5000	128F0462
Sodium Chloride	Fisher Scientific	BP358	883254
Sodium Citrate	Fisher Scientific	BP327	895942
Sodium Hydroxide	Fisher Scientific	S318	893102
Sodium Iodide	Mallinckrodt	1141	KETC
Sorbitol	Fisher Scientific	BP439	884322
StrataClean™	*StrataGene*	400713	7668
Streptomycin Sulfate	Gibco	8601860IJ	79N0800
Sucrose	Fisher Scientific	S5	874244
Tris Base	Sigma	T1503	60H-5611
Tris HCl	Fisher Scientific	BP153	880765
Tris Ultrapure	U.S. Biochemical	22643	70659
Tryptone	Difco	0123-01	759674
Urethane	Sigma	U2500	69F-0633
Xylene Cyanole FF	Sigma	X4126	80H3740
Yeast Extract	Difco	0127-01-7	774912
$ZnSO_4 \cdot 7H_2O$	Aldrich	22137-6	HX06127PW

Media

Stock Salts Sol'n	In 1 L
MgO	10.75 g
$CaCO_3$	2.0 g
$FeSO_4 \cdot 7H_2O$	4.5 g
$ZnSO_4 \cdot 7H_2O$	1.44 g
$MnSO_4 \cdot 4H_2O$	1.12 g
$CuSO_4 \cdot 5H_2O$	0.25 g
$CoSO_4 \cdot 7H_2O$	0.28 g
H_3BO_3	0.06 g
HCl (conc.)	51.3 ml

Vogel's Minimal	
Sodium Citrate	2 g/l
NH_4NO_3	1 g/l
KH_2PO_4	1 g/l
$MgSO_4 \cdot 7H_2O$	0.5 g/l
NaCl	0.1 g/l
$CaCl_2$	0.1 g/l
Biotin	2-5 mg/l
Stock Salts Sol'n	1 ml/l
Sucrose	15 g/l

PU 7

(NH4)2SO4	1.0 g/l
KH2PO4	5.0 g/l
MgSO4 • 7H2O	0.1 g/l
FeSO4 • 7H2O	5.0 mg/l
Stock Salts Sol'n	1.0 ml/l
Gel-Gro® (ICN Biochemicals)	12.0 g/l
MgCl2 • 6H2O	0.75 g/l
pH 7.0 w/ NaOH Before Autoclaving	
After Autoclaving Add Impranil® Polyurethane	2.9 ml/l

LB

Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	5 g/l
pH 7.5 w/ NaOH	

NB

Beef Extract	3.0 g/L
Peptone	5.0 g/L
pH 6.8	

U 7

Same as PU7 except add 0.2% Urethane instead of Polyurethane after autoclaving

For solid media of PU7 and U7: 12.0 g/l Gel-Gro® and 0.75 g/l $MgCl_2 \cdot 6 H_2O$ are added before autoclaving
For solid media of Vogel's Minimal and LB: 15.0 g/l of Agar are added before autoclaving

Manufacturers

Aldrich	Milwaukee, Wisconsin	800-558-9160
Baker	Phillipsburg, New Jersey	800-582-2537
Boehringer Mannheim	Indianapolis, Indiana	800-262-1640
Difco	Detroit, Michigan	800-521-0851
Fisher Scientific	Santa Clara, California	800-622-6047
FMC BioProducts	Rockland, Maine	800-342-1574
ICN Biochemicals	Costa Mesa, California	800-854-0530
Mallinckrodt	Paris, Kentucky	606-987-7000
Mobay	Pittsburgh, Pennsylvania	412-777-2000
Novo BioLabs	Danbury, Connecticut	800-344-6686
Quantum Chemical	Cincinnati, Ohio	513-530-6500
Sigma	St. Louis, Missouri	800-325-3010
Stratagene	La Jolla, California	800-424-5444
U.S. Biochemicals	Cleveland, Ohio	800-321-9322

Polyurethane and Urethane Degradation Tests

Fifteen (15) cultures were started using polyurethane (PU 7) and urethane (U 7) as the sole carbon source in a minimal salts solution. Tests were conducted with 10 ml of medium in 16 x 150 mm culture tubes at room temperature. The tubes were capped with Bellco stainless steel culture tube closures and placed in a tube rotator at a 30° angle and 80 RPM. Solid media of PU 7 and U 7, with GelGro™ as the solidifying agent, were also prepared. These were inoculated with the same cultures and incubated at 30 °C.

Inoculum cultures:

ATCC 35698	ATCC 9642	D0
ATCC 11172	HAFB-2	S1
ATCC 53922(TRA)	HAFB-2F	S2
ATCC10196	TF	P1
ATCC12668	PF	P2

TRA mixed cultures P1 & S1 growing on PU 7 medium with Gel-Gro® as the solidifying agent are making clearing zones (plaques) in the medium. The polyurethane makes the otherwise clear salts medium opaque. Four other cultures also have good growth on PU 7: HAFB-2F, 9642, TF & D0. Cultures 53922 & HAFB-2F show good growth on U 7 medium. The medium is clear, so clearing zones are not seen.

The first signs of plaque formation took almost 2 months from the date of inoculation. With subsequent tests, the rate of plaque formation is down to approximately 2 weeks. In a another test, two separate plates showed the same rate of degradation: 1) a P1 inoculum (grown on PU 7) was plated on NB medium, (grown for one week), and was then inoculated back to PU 7 medium; 2) a P1 inoculum (grown on PU 7) was plated on PU 7 medium. Both of the final PU 7 plates had the same rate of degradation. Thus the induction of polyurethane degradation was not affected by growth on another carbon source.

A bacteria and a fungus from P1 were isolated on solid media and tests are proceeding to determine whether one or both are responsible for the polyurethane degradation.

A colormetric test is being developed for isocyanate and urethane to measure the rate and extent of degradation. The test is based on a procedure in Polyurethane Elastomers by C. Hepburn which uses *p*-dimethylaminobenzaldehyde as a reagent to detect the presence of isocyanate and urethane. The reaction produces a yellow color. Using a spectrophotometer and a calibration curve, the degradation can be quantified.

Isolation of Organisms

Two fungi species were isolated on polyurethane paint selective medium (25 ml acetone + 75 ml polyurethane paint mixed/ liter NB) from Hill AFB paint waste samples. The mix of the two fungi in liquid medium removes polyurethane paint from the surface of painted balls in less than 68 hours. The two fungi were separated on solid media (NB) and grown in separate flasks as pure cultures in liquid NB. They were designated HAFB-2F-Br and HAFB-2F-Wh. They both exhibited paint stripping activity but HAFB-2F-Br had a faster rate of growth and stripping. Thus, HAFB-2F-Br was chosen to proceed with cloning.

Proteins

A one liter culture of HAFB-2F was grown. Proteins have been extracted using an $(NH_4)_2SO_4$ precipitation. The procedure is as follows: The culture was filtered through a buchner funnel using 15 cm Fisher Scientific P4 qualitative filter paper. To the filtrate 460.82 g of $(NH_4)_2SO_4$ was added to bring the filtrate to 80% salt. The solution was then spun at high speed (25,000 RPM) for 30 min. The supernatant was discarded and the pellets were resuspended in 10 ml of TES buffer(15 mM Tris ph 8, 5 mM EDTA, 100 mM NaCl). To this was added 2.31 g of $(NH_4)_2SO_4$ to bring the concentration to 40%. The solution was then centrifuged as before, the pellet was discarded and the supernatant was saved. The supernatant was then brought to 80% with the addition of 2.64 g $(NH_4)_2SO_4$. The solution was then centrifuged again and the pellet was saved and

resuspended again in TES buffer. 200 µl of this was then run on a Pharmacia FPLC (Fast Protein Liquid Chromatography) system. Using a HR 10/30 column with Superose 12, the TES elution buffer flow rate was 1.0 ml/min. The UV (blue line) detector was 280 nm and the refractive index (red line) detector was 225 nm. Chart paper speed was 0.5 cm/min (Fig.1). Five fractions were collected from the column. These fractions were run on a 15% SDS-PAGE at 70 volts overnight. The visible resolution of the gel was poor due to the low protein concentration. The five fractions will also be assayed for stripping activity.

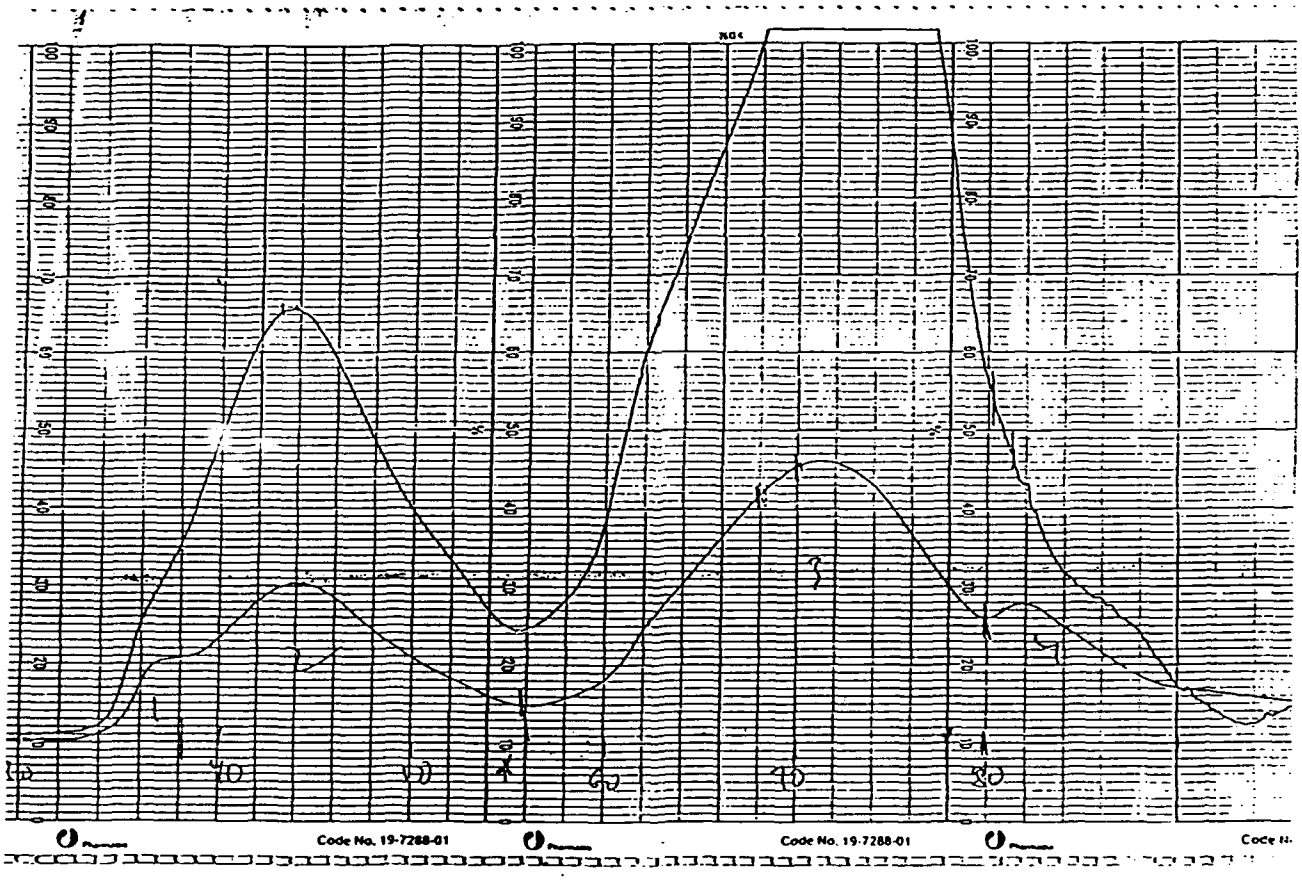


Figure 1 FPLC Column Detector Plot
Top Trace: Refractive Index 225 nm
Bottom Trace: UV Absorption @ 280 nm
Time In Minutes Along Bottom

Vector and Hosts

The DNA vector and fungal host was received from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center. They are the cosmid pSV50 (Fig 2) in an *E. coli* host, LM83, and *Neurospora crassa* wild type strain (74-OR23-1VA), FGSC # 2489. The genomic library will be constructed in *E. coli* HB101. The fungal vector host will be used for expression of the genes from HAFB-2F-Br. The cosmid in its' *E. coli* host and the *E. coli* host for the library construction is grown in LB medium. *Neurospora crassa* is grown in Vogel's medium.

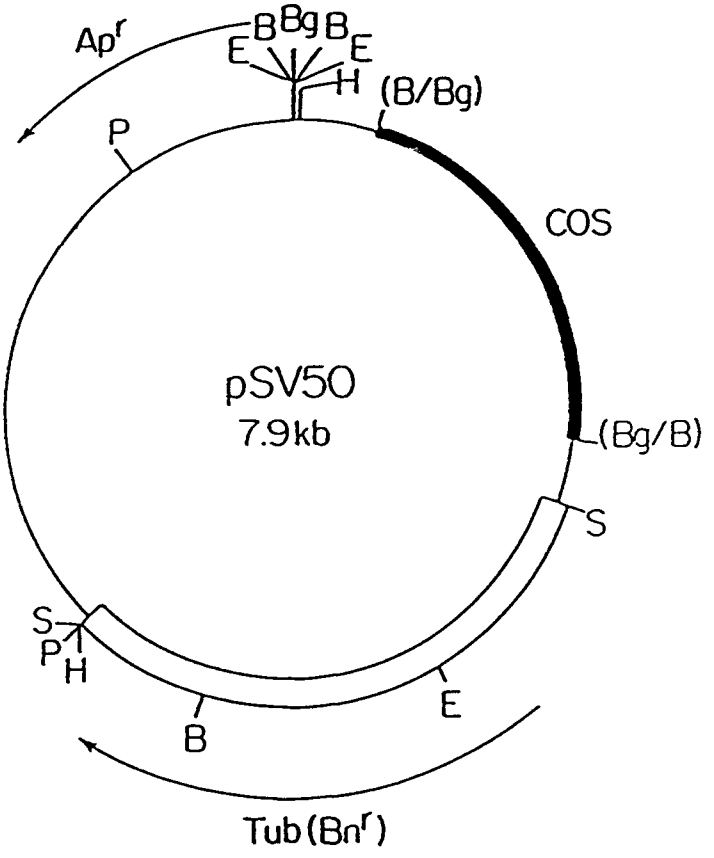


Figure 2 Cosmid pSV50
Restriction Enzyme Sites: B: *Bam HI*; Bg: *Bgl II*; E: *Eco RI*; H: *Hind III*; P: *Pst I*; S: *Sal I*
Tub(Bn^r): Benomyl Resistance
Ap^r: Ampicillin Resistance

Plasmid Miniprep

A miniprep is a small scale plasmid purification procedure used to isolate plasmid DNA from its' bacterial host. The procedure used for isolation of pSV50 is a modification of the standard alkali lysis procedure found in Sambrook and is as follows:

After growing the plasmid host overnight in LB medium in either 15 ml or 50 ml centrifuge tubes, the test tube was spun down at 2500 x g for 10 min. The supernatant was discarded and the pellet was transfered to an Eppendorf tube and resuspended in 400 µl of 10 mg/ml of lysozyme in solution I (50 mM Glucose, 10 mM EDTA and 25 mM Tris HCl pH 8.0). The mixture was allowed to digest at room temperature for 5 min. 400 µl of solution II was added (0.2 N NaOH and 1% SDS). The solution was mixed gently and placed on ice for 25 min. 600 µl of 3M $KC_2H_3O_2$ was added and the mixture was placed on ice for 45 min. The mixture was then spun for 5 min. and the supernatant transfered to a new tube to which 1x volume of isopropanol was added. The mixture was allowed to precipitate at room temperature for 15 min., after which it centrifuged for 15 min. to pellet the plasmid. The supernatant was discarded and the pellet was resuspended in 200 µl of DI H2O. 100 µl of 3M $NH_4C_2H_3O_2$ was added to precipitate the RNA. This solution was allowed to stand at room temperature for 15 min., after which the RNA was isolated by centrifuging for 5 min. The pellet was discarded and 1x volume of isopropanol was added to the supernatant. The solution was left at room temperature for 15 min. This solution was then centrifuged for 10 min. The pellet was rinsed with 70% ethanol and dried carefully with a Kimwipe. The pellet was then resuspended in 20 µl of DI H2O and stored at -20 °C.

Digestion of Plasmid

The pSV50 plasmid (cosmid) was digested with *Bgl II* to remove the *cos* site (which is not needed for our use) and to reduce the number of *Bgl II* sites in the plasmid to just one. pSV50 was chosen for its' ability to transform both *E. coli* and *Neurospora crassa*. *E. coli* will be used for maintaining the genomic library and *Neurospora crassa* will be used for screening the library.

From the above miniprep, 15 µl of plasmid DNA was digested with 3 µl of *Bgl II* restriction enzyme in 4 µl of Boehringer Mannheim's 10x buffer "M" and 33 µl DI H2O at 37°C for 2 hrs. The digest was then run on a 0.7% low-melting agarose gel in TAE (50x= 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA ph 8.0)

Three bands were visualized under UV light: The top band being undigested pSV50, the middle one was the modified plasmid pSV50* (≈6.1 kb) and the bottom band was the *cos* site fragment (≈1.8 kb). The pSV50* band was cut out from the gel. The DNA was extracted from the agarose using a SpinBind™ DNA Extraction Unit (FMC BioProducts) following the protocol included with the product.

Ligation

The extracted linear plasmid DNA was then ligated to itself to form a circular plasmid. 25 µl of extracted DNA was ligated using 1 µl of T4 DNA Ligase, 3 µl 10x ligation buffer and 1 µl DI H_2O. The mixture was placed in a water bath at 16 °C and allowed to react overnight. Some of the plasmid was stored in its' linearized form. This will eliminate the need for re-digesting the plasmid with *Bgl II* before cloning.

Preparing Competent Cells

Competent cells are cells which are chemically treated to make them more capable of taking up foreign, extracellular DNA. Competent cells were prepared with the following procedure:

A 25 ml culture of *E. coli* (Strain SU1675 Kan^R) was started in LB medium with 40 µg/ml Kanamicin and grown to an optical density of 0.6 - 0.8 at 600 nm. The cells were then chilled on ice for 5 min. and then centrifuged for 5 min. at 10,000 RPM. The cells were then resuspended in 2 ml of 0.1 M $CaCl_2$. The cells were then frozen and stored in liquid nitrogen vapor phase until needed.

Transformation

Transformation is the process of introducing extracellular DNA (ie: a plasmid) into a competent cell. Transformation of *E.coli* was performed as follows:

500 µl of competent cells were thawed on ice. After thawing, 30 µl of ligated plasmid DNA was added and the solution was left on ice for 30 min. The mixture was then heat-shocked at 42°C for 2 min. and then spread plated onto LB medium with Ampicillin (50 µg/ml). The pSV50* plasmid carries the Amp^R gene so that only successfully transformed *E. coli* cells will grow.

Transformation of *Neurospora crassa* will be more difficult than *E coli*. There are published protocols on transforming fungi by

chemical methods which involve generating protoplasts. The transformation frequency for these procedures however, is usually low. Electroporation will, therefore, be used. Electroporation utilizes high voltage (500 - 2500 V) with controled discharge rates (τ = 5 μsec - 1 sec) to introduce extracellular DNA within the cells. Intact fungi can be used, thus eliminating the protoplast formation step.

Isolation of HAFB-2F-Br Genomic DNA

Several different procedures have been used to extract the genomic DNA from the organism. The methods utilizing lysing enzymes, Gluculase and Novozyme, digest the mycelium and produce protoplasts but also tend to digest the DNA. The detergent procedures did not work well initially. A procedure was modified to include freezing the mycelium with liquid nitrogen and blending in a stainless steel blender. This improved the yield of DNA and did not degrade it.

Gluculase Procedure

One liter of fresh HAFB-2F-Br culture, grown to saturation, was vacuum filtered through Whatman filter paper #40, yielding between 0.5 - 5 g of wet mycelium. The filtrate was then ground under liquid nitrogen to a coarse powder in a pre-cooled Waring blender and resuspended in TE (10 mM Tris pH 7.6, 1 mM EDTA) at 0.25 g filtrate/ml TE in 15 ml centrifuge tubes. Gluculase was added at 3% with 2.5 μl 1 M sorbitol and the prep was incubated at 30 °C for 15 - 18 hours. After checking under a microscope to insure that the mycelia had been digested, SDS was added at 100 μl/ml. The tubes were then gently rocked until the mixture became viscous. The prep was then incubated at 37 °C for one to two hours. Then the prep was centrifuged in 15 ml conical tubes at 3850 RPM for 5 minutes and the supernatant was transferred to clean tubes. To remove any RNA, Ribonuclease A was added at 10 μg/ml and the prep was allowed to digest at 37 °C for one hour. The protein was removed by adding an equal amount of tris-saturated phenol, shaking the tubes for 30 seconds and centrifuging for five minutes at 3850 RPM. The aqueous layer was retained. This step was repeated approximately four to nine times until the aqueous phase was reasonably clean and no protein appeared in the interface. Any traces of phenol remaining in the aqueous layer were removed by adding an equal amount of chloroform/isoamyl alcohol (24:1), shaking, and centrifuging as above. The DNA was then precipitated

by adding two volumes of absolute ethanol, chilling at -20 °C for at least 15 minutes and centrifuging in 1.7 ml eppendorf tubes at 10400 RPM for 10 minutes. The supernatant was poured off, and remaining liquid was removed from the sides of the tubes with a Kimwipe. The tubes were inverted and pellets were allowed to dry completely, after which they were resuspended in 50-100 µl TE and stored at -20 °C.

Novozyme Procedure

One liter of fresh HAFB-2F-Br culture, grown to saturation, was vacuum filtered through Whatman filter paper, #40, yielding between 0.5 - 5 g of wet mycelium. The filtrate was then ground under liquid nitrogen to a coarse powder in a precooled Waring blender and resuspended in buffer (15% sucrose, 50 mM Tris pH 7.6, 50 mM EDTA) at 0.2 g filtrate/ml buffer in 15 ml centrifuge tubes. Novozyme was added at 40 mg/ml and the prep was incubated at 30 °C for 15 - 18 hours. After checking under a microscope to insure that the mycelia had digested, SDS was added at 100 µl/ml. The tubes were gently rocked until the mixture became viscous. The prep was then incubated at 37 °C for one to two hours. Then the prep was centrifuged in 15 ml conical tubes at 3850 RPM for 5 minutes and the supernatant was transferred to clean tubes. To remove any RNA, Ribonuclease A was added at 10 µg/ml. The prep was allowed to digest at 37 °C for one hour. The protein was removed by adding an equal amount of tris-saturated phenol, shaking the tubes for 30 seconds and centrifuging for five minutes at 3850 RPM. The aqueous layer was retained. This step was repeated approximately four to nine times until the aqueous phase was reasonably clean and no protein appeared in the interface. Any traces of phenol remaining in the aqueous layer were removed by adding an equal amount of chloroform/isoamyl alcohol (24:1), shaking, and centrifuging as above. The DNA was then precipitated by adding two volumes of absolute ethanol, chilling at -20 °C for at least 15 minutes and centrifuging in 1.7 ml eppendorf tubes at 10400 RPM for 10 minutes. The supernatant was poured off, and remaining liquid was removed from sides of the tubes with a Kimwipe. The tubes were inverted and pellets were allowed to dry completely, after which they were resuspended in 50-100 µl TE and stored at -20 °C.

Blin and Stafford - Based Procedure

In a trial preparation, mycelia were harvested by vacuum filtration onto a Whatman filter paper. 100 ml of fungal culture provided 0.5 g of mycelia. The mycelia were removed from the filter paper and ground by mortar and pestle under liquid nitrogen for 15 to 20 minutes until they were completely pulverized. This powder was then transferred to a 15 ml Falcon tube and suspended in 2 ml of extraction buffer (0.5 M EDTA, 0.5% n-lauryl sarcosine (Sarkosyl)). After vortexing to suspend the powder, proteinase K was added at 100 µg/ml. The tube was agitated for 3 hours in a 50 °C water bath.

The preparation was removed from the water bath and diluted with 1x volume of DI H_2O. It was then extracted 3 times with a 20:3 mixture of phenol:m-cresol. Between extractions, the tube was spun at 3800 RPM to separate the phases. The aqueous phase was removed to a fresh tube.

In order to remove all traces of phenol, the aqueous phase was placed into a dialysis bag, which had been pre-treated by boiling for 5 minutes in 0.1 M EDTA. The DNA was dialyzed for 3 days with 3 changes (2 L each) of buffer (50 mM Tris, pH 8, 10 mM EDTA, 10 mM NaCl) until phenol could no longer be smelled in the solution.

The DNA solution was emptied from the dialysis bag into a clean 50 ml Falcon tube containing heat-treated RNase A at a final concentration of 100 µg/ml. The reaction was left at 37 °C for 3 hours.

Again the solution was extracted twice with phenol and dialyzed against the same buffer for 3 days through 3 changes of buffer. Finally, the DNA was precipitated in 0.6 volumes of isopropanol by standing overnight at room temperature. The DNA was spun down at 3800 RPM and washed once with 70% ethanol. The yield was extremely poor.

SDS Procedure

A one liter culture of fungus was grown for several days in NB (50 U/ml pennicillin, 50 mg/ml streptomycin) and then vacuum filtered onto a Whatman filter paper to yield approximately 3 to 5 g of wet mycelia. The mycelia were peeled as a mat from the filter paper and ground under liquid nitrogen for 5 to 10 minutes in a stainless steel Waring blender until they were finely powdered. After allowing the liquid nitrogen to evaporate, the powder was transferred into 15 ml Falcon tubes such that each tube contained between 0.5 and 1 g of the powdered fungus. To each tube was then

transferred into 15 ml Falcon tubes such that each tube contained between 0.5 and 1 g of the powdered fungus. To each tube was then added the extraction buffer (50 mM Tris, pH 8, 100 mM EDTA, 250 mM NaCl, 1% SDS) at about 2 ml/0.5 g of fungus. When the tubes had been vortexed in order to completely suspend and disperse the mycelia powder, 100 µl/ml of SDS was added to each tube from a 20% stock solution. After mixing again, the tubes were incubated at 65 °C for 3 hours.

Following the incubation, the tubes were spun at 3850 RPM for 30 minutes to pellet out the cell debris. The supernatants were poured into clean Falcon tubes and extracted once with a 3:1 mixture of phenol:chloroform. The tubes were spun again for 15 minutes to separate the phases. The aqueous phase was then collected and extracted twice with a 24:1 mixture of chloroform:isoamyl alcohol. The aqueous phase was brought to a high salt concentration by the addition of 1/10 its volume of 3 M $NaC_2H_3O_2$ (pH 5.2). Twice its volume of ice cold absolute ethanol was added to precipitate the DNA. The tube was left at -20 °C for 20 minutes to allow all the DNA to precipitate, and then the tube was spun at 3850 RPM for 30 minutes to pellet the DNA. The pellet was washed once with 70% ethanol to remove excess salts and then the DNA was resuspended in a small volume of TE buffer.

Partial Restriction Digest of Genomic DNA

The isolated genomic DNA from a Novozyme 234 prep was partially digested with *Sau 3A* to create small fragments suitable for cloning. 50 µl of Genomic DNA from the SDS isolation was mixed with 6 µl of buffer A and 4 µl *Sau 3A* (12 units) in a microcentifuge tube. The reaction was incubated at 37 °C. 12 µl aliquots were removed every 15 minutes for the first hour of incubation. To each aliquot removed, 10 µl of running buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15 % Ficoll) was added and the sample placed on ice in a microcentifuge tube. The remaining reaction mixture was incubated for another hour, 2 hours total, after which 10 µl of running buffer was added and placed on ice. The 5 samples were then run on a 0.6% agarose gel to separate the DNA by size in TBE buffer (5x sol'n= 54 g Tris, 27.5 g boric acid, 20 ml 0.5 M EDTA pH 8.0). Upon comparing the digested lanes (15', 30', 45', 60', 120') to the undigested lane (control), it appeared that the restriction enzyme did not cut the DNA.

Restriction enzymes cut double stranded DNA at sequences specific to a given enzyme. *Sau 3A*, for example, recognizes the base

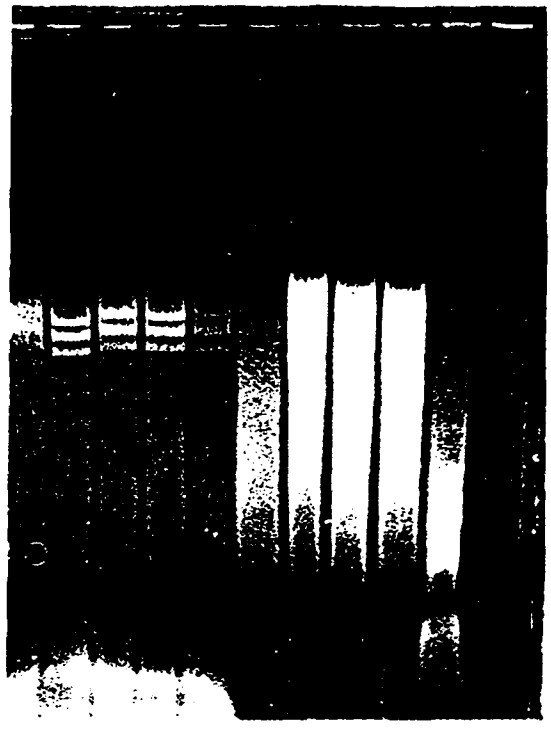
sequence GATC (5'-3') and cuts the sugar-phosphate backbone of the DNA helix to the left (5' side) of the G: N/GATC, where N is any of the four bases. The 3'-5' strand would also be cut to the 5' side of the G: CTAG/N

In order to test whether the above result was due to a bad enzyme or contamination of the genomic DNA, we ran the following digests:

Lane (Fig 3)
1) undigested genomic DNA (HAFB-2F-Br)
2) *Bam HI* digested genomic DNA (HAFB-2F-Br)
3) *Bgl II* digested genomic DNA (HAFB-2F-Br)
4) *Hind III* digested genomic DNA (HAFB-2F-Br)
5) *Sau 3A* digested genomic DNA (HAFB-2F-Br)
6) undigested calf-thymus DNA
7) *Bam HI* digested calf-thymus DNA
8) *Bgl II* digested calf-thymus DNA
9) *Hind III* digested calf-thymus DNA
10) *Sau 3A* digested calf-thymus DNA
11) Calf-thymus DNA incubated 2 hrs. with 10 mg Novozyme 234

The reactions used 10 μg of DNA and 18 units of enzyme for each digestion except *Sau 3A* which had 15 units of enzyme. Incubation was at 37 °C for 2 hours. They were then run on an agarose gel as before. The genomic DNA did not appear to be digested but there were 3 bands resolved in the lanes. These could be plasmids from the fungus.

From the reactions using calf-thymus DNA, it appears that *Bam HI, Bgl II, and Hind III* are not cutting well but *Sau 3A* is cutting well. Since *Sau 3A* cut the calf-thymus DNA and not the genomic DNA from HAFB-2F-Br, we believe that there are still proteins bound to the DNA. These proteins are preventing the restriction enzymes from cutting the DNA by blocking access to the DNA. Thus, the DNA must be purified further.



1 2 3 4 5 6 7 8 9 10 11

Figure 3 Restriction Digests

On Going Work

Work is proceeding on further purification of the HAFB-2F-Br genomic DNA. Strataclean™ resin from Stratagene will be used to purify the DNA both with and without phenol extraction.

An electroporation device is under construction. Upon its' completion we will begin experiments to optimize the transformation frequency for *Neurospora crassa* using pSV50*.

We will perform plasmid isolation on HAFB-2F-Br using plasmid minipreps and cesium chloride density gradient ultracentrifugation. If one or more plasmids are isolated, we will use them to transform *Neurospora crassa* and then screen for paint stripping activity.

Dr. Blake is attempting to isolate the protein(s) responsible for the paint striping activity. If he is successful, he will then sequence the protein(s). This will make it possible to isolate the gene(s) of interest by making degenerate oligonucleotides to to probe a cDNA library.

The protein fractions previously isolated will be assayed for paint stripping activity. This will narrow the search for the active protein(s) by identifying the size range.

References

1) Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
2) Darby, R.T. and Kaplan, A.M., Applied Microbiology, 16: 900: 1968.
3) Huang, S.J. and Roby, N.S., J. Bioact. & Compat. Polym., 1: 61: 1986.
4) Kaplan, A.M., Darby,R.T., Greenberger, M. and Rogers, M.R., Developments In Industrial Microbiology, 9: 201: 1968.
5) Mark-Bikales-Overberger-Menges, Encyclopedia of Polymer Science and Engineering, Vol 2, 2nd Ed., John Wiley and Sons, New York, 1985.
6) Economic Microbiology, Vol. 6, ed. by Rose A.H., Academic Press, London, 1981.
7) Vollmer, S.J. and Yanofsky, C., Proc. Natl. Acad. Sci. USA, 83: 4869: 1986.
8) Chung, C.T. and Miller, R.H., Nucleic Acids Res., 16: 3586: 1988.
9) Stohl, L.L. and Lambowitz, A.M., Proc. Natl. Acad. Sci. USA, 80: 1058: 1983.
10) Schweizer, M. et al, Proc. Natl. Acad. Sci. USA, 78: 5086: 1981.
11) Garber, R.C. and Yoder, O.C., Anal. Biochem., 135: 416: 1983.
12) Garber, R.C., Turgeon, B.G. and Yoder, O.C., Mol. Gen. Genet., 196: 301: 1984.
13) Blin, N. and Stafford, D.W., Nucleic Acids Res., 3: 2303: 1976.
14) Hepburn, C., Polyurethane Elastomers, Applied Science Publishers, New York, 1982.

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